Effects of Exogenous NO Treatment on Aluminum Stress Resistance in Four Species of Eucalyptus Seedlings: Postprint

Authors: Liu Bing, Guo Rongkun, Shi Maoxin, Luo Yishan, Jiang Fengjing, Teng Weichao

Date: 2023-05-21T00:00:00+00:00

Abstract

To investigate the effects of exogenous nitric oxide (NO) on aluminum tolerance in eucalyptus seedlings under aluminum stress, four species of three-month-old eucalyptus seedlings (Eucalyptus grandis, Eucalyptus urophylla, Eucalyptus tereticornis, and Eucalyptus urophylla × E. grandis) were used as experimental materials, with sodium nitroprusside (SNP) as the exogenous NO donor, using a hydroponic method to examine the effects of different NO concentrations (0, 50, 100, 200, 400, 800 $\text{mol} \cdot \text{L}^{-1}$) on physiological indices such as ROS, antioxidant enzyme activities, and organic osmotic adjustment substance contents in eucalyptus seedlings under 120 $\text{mg} \cdot \text{L}^{-1}$ aluminum stress, and to compare the differences in aluminum tolerance among the four eucalyptus species under NO treatment. The results showed that: (1) Under aluminum stress, the addition of appropriate concentrations of exogenous NO ($50 \ \text{mol} \cdot \text{L}^{-1} \leq \text{NO} \leq 200 \ \text{mol} \cdot \text{L}^{-1}$) promoted increases in soluble sugar and soluble protein contents, antioxidant enzyme (SOD, POD, CAT, APX) activities, scavenging of ROS, and reduction of MDA accumulation, thereby enhancing aluminum resistance in the four eucalyptus species, whereas under high NO concentration ($\geq 800 \ \text{mol} \cdot \text{L}^{-1}$) treatment, antioxidant enzyme activities and osmotic adjustment substance contents decreased, exhibiting stress responses. (2) Nitric oxide had a strong enhancing effect on aluminum tolerance in aluminum-sensitive eucalyptus, while the enhancement was not significant in aluminum-tolerant eucalyptus, ultimately leading to convergence of aluminum resistance among the four eucalyptus species under NO treatment. (3) Indices such as SOD, MDA, CAT, ${O_2}^{-}$, soluble protein, and soluble sugar can serve as key indicators for evaluating aluminum tolerance in eucalyptus. This study provides scientific reference for the selection of aluminum-tolerant eucalyptus germplasm resources and lays a foundation for understanding the mechanisms by which NO regulates interspecific differences in aluminum tolerance in eucalyptus.

Full Text

Preamble

Effects of Exogenous NO Treatment on Aluminum Stress Resistance in Four Eucalyptus Species

LIU Bing[1,2,3,4,5], GUO Rongkun[1], SHI Maoxin[6], LUO Yishan[1], JIANG Fengjing[1], TENG Weichao[1,2,3,4,5,*]

[1]College of Forestry, Guangxi University, Nanning 530004, China
[2]Guangxi Key Laboratory of Forest Ecology and Conservation, Nanning 530004, China
[3]State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Nanning 530004, China
[4]Key Laboratory of National Forestry and Grassland Administration for Fast-Growing Tree Breeding and Cultivation in Central and Southern China, Nanning 530004, China
[5]Guangxi Colleges and Universities Key Laboratory for Forestry Science and Engineering, Nanning 530004, China
[6]Guangxi Dongmen Forest Farm, Chongzuo 532108, Guangxi, China

Abstract

This study investigated the effects of exogenous nitric oxide (NO) on aluminum tolerance in Eucalyptus seedlings under aluminum stress. Using sodium nitroprusside (SNP) as an NO donor, we employed hydroponic culture to examine the impacts of different NO concentrations (0, 50, 100, 200, 400, and 800 $\mathrm{mol}\cdot\mathrm{L}^{-1}$) on physiological indicators including reactive oxygen species (ROS), antioxidant enzyme activities, and organic osmotic adjustment substance contents in four three-month-old Eucalyptus species (*Eucalyptus grandis*, *E. urophylla*, *E. tereticornis*, and *E. urophylla* × *E. grandis*) under 120 $\mathrm{mg}\cdot\mathrm{L}^{-1}$ aluminum stress. We also compared differences in aluminum resistance among the four species under NO treatment. The results showed that: (1) Under aluminum stress, supplementation with appropriate concentrations of exogenous NO ($50\ \mathrm{mol}\cdot\mathrm{L}^{-1} \leq \mathrm{NO} \leq 200\ \mathrm{mol}\cdot\mathrm{L}^{-1}$) promoted increases in soluble sugar and soluble protein contents, enhanced antioxidant enzyme activities (SOD, POD, CAT, APX), facilitated ROS scavenging, reduced MDA accumulation, and improved aluminum resistance in all four species. However, at high NO concentrations ($\geq 800\ \mathrm{mol}\cdot\mathrm{L}^{-1}$), antioxidant enzyme activities and osmotic adjustment substance contents decreased, exhibiting stress responses. (2) Nitric oxide significantly improved aluminum tolerance in sensitive Eucalyptus species but had less pronounced effects on tolerant species, ultimately resulting in similar aluminum resistance across all four species under NO treatment. (3) SOD, MDA, CAT, $O_2{}^-$, soluble protein, and soluble sugar can serve as key indicators for evaluating aluminum tolerance in Eucalyptus. This study provides a scientific reference for selecting aluminum-tolerant Eucalyptus germplasm resources and lays a foundation for understanding the mechanisms underlying NO regulation of interspecific differences in aluminum tolerance.

Keywords: Eucalyptus, exogenous NO, aluminum stress, physiological indicators, principal component analysis, aluminum resistance

Introduction

Aluminum is the most abundant metal element in the Earth's crust. Aluminum toxicity induces the generation of large amounts of reactive oxygen species (ROS) in plants, causing oxidative stress that increases cell membrane permeability (Pereira et al., 2011). Addressing aluminum toxicity to effectively utilize acidic soil resources has become a significant concern for soil and plant scientists. Eucalyptus (*Eucalyptus spp.*), belonging to the family Myrtaceae and genus *Eucalyptus* (Xie Yaojian, 2015), has a long cultivation history, rapid growth, wide adaptability, and high yield, occupying an important position in China's forestry industry (Huang Liping et al., 2022; Wei Yihui et al., 2021). Eucalyptus plantations are primarily distributed in southern China, where soils are acidic and highly weathered, with abundant aluminum and iron content (Huang Qianqian, 2021) that inhibits Eucalyptus growth and development, severely affecting yield and quality.

Nitric oxide (NO) is an important redox signaling molecule that regulates plant growth and development and transmits signals to enhance stress resistance when plants are under stress. However, NO may also act as a reactive nitrogen species that accumulates in large quantities within plants, causing nitrosative stress and damaging plants (Li Yan, 2017). Recent research has explored NO's role in alleviating aluminum toxicity in plants, with several mechanisms identified: (1) enhancing antioxidant capacity (González et al., 2012); (2) reducing heavy metal accumulation in plants (Xiong et al., 2009); and (3) regulating expression of metal resistance-related genes (Xiong et al., 2010). Studies have shown that NO reduces aluminum-induced oxidative damage and improves aluminum resistance in *Phoebe bournei*, soybean, and tobacco by regulating osmotic substances and increasing antioxidant enzyme activities (Li Lin, 2020; Wang Huahua et al., 2019; Liu Qiang et al., 2017). Both hydrogen peroxide (H_2O_2) and NO are small molecular signaling substances with dual physiological functions of toxicity and cell protection (Yu et al., 2014). Under stress conditions, the effects of endogenous H_2O_2 and exogenous NO on antioxidant system metabolism play crucial roles in plant stress responses (Yin et al., 2010). However, the effects of exogenous NO on H_2O_2 metabolism and antioxidant system responses in Eucalyptus under aluminum stress have not been reported and warrant in-depth investigation.

To better understand NO's influence on aluminum resistance mechanisms in Eucalyptus species with different aluminum tolerances, we selected four Eucalyptus species with significantly different aluminum tolerances identified in our previous research: pure species *Eucalyptus grandis*, *E. tereticornis*, *E. urophylla*, and hybrid *E. urophylla* × *E. grandis* (Liang Yanhong, 2022; Li Tangkan, 2020). Among these, *E. grandis* and *E. tereticornis* are aluminum-sensitive, while *E. urophylla* and *E. urophylla* × *E. grandis* are aluminum-tolerant. Using hydroponic culture, we measured and analyzed the effects of different NO concentrations on ROS, antioxidant enzyme activities, osmotic adjustment substances, and membrane lipid peroxidation indicators in Eucalyptus seedlings under aluminum stress. We aimed to address: (1) how physiological indicators in leaves of Eucalyptus species with different aluminum tolerances change under aluminum stress and their relationship with aluminum tolerance; (2) how exogenous NO treatment affects differences in aluminum tolerance among the four Eucalyptus species; and (3) what NO concentration ranges are beneficial for improving aluminum resistance in each of the four species. The results will provide theoretical references for improving aluminum tolerance in Eucalyptus seedlings in acidic soils and for breeding and utilizing aluminum-tolerant Eucalyptus germplasm resources, offering guidance for high-quality and high-yield cultivation of Eucalyptus in aluminum-contaminated soil areas.

1.1 Experimental Materials

The experimental materials consisted of healthy, uniformly growing three-month-old Eucalyptus seedlings (*E. grandis*, *E. urophylla*, *E. tereticornis*, and *E. urophylla* × *E. grandis*) provided by the Forest Science Research Institute of Guangxi Dongmen Forest Farm (107°84 E, 22°17 N). After transport to the College of Forestry, Guangxi University (108°17 9.00 E, 22°50 28.41 N, subtropical monsoon climate with annual average temperature of 22.6°C and annual rainfall of 1,100–1,300 mm), the seedlings were cultivated hydroponically outdoors from April 10 to April 24, 2022. The hydroponic method followed Lu Mingying (2014). Soil was gently removed from seedling roots, which were then rinsed with tap water, disinfected in 1‰ carbendazim solution for 20 minutes, and rinsed again. Plants were secured on fully opaque pearl cotton foam boards cut to fit black plastic buckets (17.8 cm × 17.9 cm). Seedlings were evenly fixed on the foam boards and placed in black plastic buckets containing 2.5 L of Hoagland nutrient solution (pH 5.5) with 0.5 $\mathrm{mmol \cdot L^{-1}}$ $CaCl_2$ (formulation shown in Table 1). Oxygen pumps were connected to provide continuous 24-hour aeration (see Figure 1 [Figure 1: see original paper] for the complete setup). The nutrient solution was replaced every three days. After one week of hydroponic culture, the pH of fresh nutrient solution was gradually adjusted to 4.5 using 1 $\mathrm{mol \cdot L^{-1}}$ HCl and NaOH solutions. After 14 days of hydroponic culture, uniformly vigorous seedlings were selected for treatment.

1.2 Experimental Design

The experiment was conducted outdoors at the College of Forestry, Guangxi University using a completely randomized design. Treatments were applied from April 24 to April 26, 2022. Healthy, uniformly growing seedlings (height 30$±10*cm*, *grounddiameter*5±2*mm*)*wereselectedfromthehydroponicculture*.*AlCl*{*3*}·7*H*{2}*Owasusedasthe* source and SNP as the NO donor. Seven treatments were established for each Eucalyptus species (Table 2), with the aluminum concentration of 120 $mg \cdot L^{-1}$ based on preliminary experiments (Liang Yanhong, 2022; Li Tangkan, 2020; Liang Junxia, 2020; Liang et al., 2022). Each treatment had three replicates (three pots) with six seedlings per replicate (six seedlings/pot), totaling 126 seedlings per species and 504 seedlings across all four species. After 48 hours of oxygen pump treatment, middle-position leaves were collected from seedlings and stored at -80°C for subsequent indicator measurement.

1.3 Measurement Indicators and Methods

MDA content was measured using the thiobarbituric acid method, soluble protein content using the Coomassie brilliant blue G-250 method, and soluble sugar content using the anthrone colorimetric method. Ascorbate peroxidase (APX) activity was determined by the vitamin C oxidation method (Wang Xuekui, 2000; Li Hesheng, 2000). Superoxide dismutase (SOD) activity was measured by the nitroblue tetrazolium method, peroxidase (POD) activity by the guaiacol method, and catalase (CAT) activity by the ultraviolet absorption method (Chen Jianxun and Wang Xiaofeng, 2006). Superoxide anion (O_2^-) production rate was determined by the hydroxylamine oxidation reaction method (Kong Xiangsheng and Yi Xianfeng, 2008), and hydrogen peroxide (H_2O_2) content was measured by the titanium sulfate colorimetric method (Yi et al., 2015).

1.4 Data Processing

Experimental data were organized using Excel 2016 and expressed as means ± standard deviation of three replicates. Duncan's multiple range test ($P < 0.05$) was performed using SPSS 26.0 software. Bar charts were created using Sigmaplot 12.0, and principal component analysis was conducted using R (R Core T, 2020). To better compare the response differences of the four Eucalyptus species to Al and SNP treatments and balance their differences in the original state (CK, no Al or SNP treatment), relative physiological indicators were used to reflect their response degrees to Al and SNP treatments, calculated as follows (Liang et al., 2022):

Relative value of T1 = Measured value of T1 treatment / Measured value of CK group
Relative values of T2–T6 = (Measured value of each T2–T6 treatment) / Measured value of T1 treatment

2.1 Effects of Treatments on O_2^- Production Rate, H_2O_2 Content, and MDA Content in Eucalyptus Seedlings

As shown in Figure 2 [Figure 2: see original paper], compared with CK, aluminum stress treatment (T1) significantly increased O_2^- production rate in *E. grandis* but had no significant effect on the other three species. With increasing SNP concentration, O_2^- production rates in leaves of all four Eucalyptus species showed a trend of first decreasing then increasing. *E. grandis* reached its minimum at T2, decreasing significantly by 3.48% compared with T1 ($P < 0.05$). *E. urophylla* reached its minimum at T3, decreasing significantly by 3.79% ($P < 0.05$). *E. tereticornis* reached its minimum at T4, decreasing significantly by 5.67% ($P < 0.05$). *E. urophylla* × *E. grandis* reached its minimum at T2, decreasing significantly by 1.94% ($P < 0.05$). All four species showed relatively low O_2^- production rates at T2, T3, and T4, indicating that appropriate SNP concentrations help reduce O_2^- production in Eucalyptus.

Compared with CK, T1 significantly increased H_2O_2 accumulation in *E. urophylla* seedlings but had no significant effect on the other three species. With increasing SNP concentration, H_2O_2 content in leaves of all four species showed a trend of first decreasing then increasing. *E. grandis* reached its minimum at T3, decreasing significantly by 13.50% compared with T1 ($P < 0.05$). *E. urophylla* reached its minimum at T5, decreasing significantly by 23.77% ($P < 0.05$). *E. tereticornis* reached its minimum at T4, decreasing significantly by 4.69% ($P < 0.05$). *E. urophylla* × *E. grandis* reached its minimum at T4, decreasing significantly by 13.32% ($P < 0.05$). All four species showed relatively low H_2O_2 contents at T3, T4, and T5, indicating that appropriate SNP concentrations help reduce H_2O_2 content in Eucalyptus.

Compared with CK, MDA content showed no significant change in *E. urophylla* under T1 but increased significantly in the other three species. With increasing SNP concentration, MDA content in leaves of all four species generally showed a trend of first decreasing then increasing. *E. grandis* reached its minimum at T3, decreasing significantly by 54.29% compared with T1 ($P < 0.05$). *E. urophylla* reached its minimum at T3, decreasing significantly by 36.03% ($P < 0.05$). *E. tereticornis* reached its minimum at T4, decreasing significantly by 40.81% ($P < 0.05$). *E. urophylla* × *E. grandis* reached its minimum at T3, decreasing significantly by 65.57% ($P < 0.05$). All four species reached their minimum values at either T3 or T4, indicating that appropriate SNP concentrations help reduce MDA content in Eucalyptus.

2.2 Effects of Treatments on Antioxidant Enzyme Activities in Eucalyptus Seedlings

As shown in Figure 3 [Figure 3: see original paper], compared with CK, T1 significantly increased SOD activity in *E. tereticornis* seedlings. With increasing SNP concentration, SOD activity in leaves of all four species generally showed a trend of first increasing then decreasing. *E. grandis* reached maximum activity at T3, increasing significantly by 9.14% compared with T1 ($P < 0.05$). *E. urophylla* reached maximum activity at T5, 2.22 times that of T1. *E. tereticornis* reached maximum activity at T4, 1.82 times that of T1. *E. urophylla* × *E. grandis* reached maximum activity at T4, 1.86 times that of T1. All four species generally reached maximum values at T3, T4, or T5, indicating that appropriate SNP concentrations help increase SOD activity.

Compared with CK, T1 significantly increased POD activity in *E. grandis* and *E. tereticornis* seedlings but had no significant effect on *E. urophylla* and *E. urophylla* × *E. grandis*. With increasing SNP concentration, POD activity in *E. urophylla* × *E. grandis* showed no significant change, while the other three species showed a trend of first increasing then decreasing. *E. grandis* reached maximum activity at T3, increasing significantly by 53.06% compared with T1 ($P < 0.05$). *E. urophylla* reached maximum activity at T5, increasing significantly by 66.04% ($P < 0.05$). *E. tereticornis* reached maximum activity at T4, increasing significantly by 50.00% ($P < 0.05$). *E. urophylla* × *E. grandis* reached maximum activity at T6, with no significant difference from T1 ($P > 0.05$), indicating that appropriate SNP concentrations help increase POD activity.

Compared with CK, T1 significantly increased CAT activity in *E. tereticornis* seedlings. With increasing SNP concentration, CAT activity in leaves of all four species showed a trend of first increasing then decreasing. *E. grandis* reached maximum activity at T3, with no significant difference from T1 ($P > 0.05$). *E. urophylla* reached maximum activity at T2, 3.72 times that of T1. *E. tereticornis* reached maximum activity at T6, increasing significantly by 41.11% compared with T1 ($P < 0.05$). *E. urophylla* × *E. grandis* reached maximum activity at T3, 3.08 times that of T1, indicating that appropriate SNP concentrations help increase CAT activity.

Compared with CK, T1 significantly increased APX activity in *E. grandis* and *E. urophylla* seedlings but had no significant effect on *E. tereticornis* and *E. urophylla* × *E. grandis*. With increasing SNP concentration, APX activity in leaves of all four species showed a trend of first increasing then decreasing. *E. grandis* reached maximum activity at T3, increasing significantly by 25.33% compared with T1 ($P < 0.05$). *E. urophylla* reached maximum activity at T5, increasing significantly by 33.59% ($P < 0.05$). *E. tereticornis* reached maximum activity at T5, increasing significantly by 54.24% ($P < 0.05$). *E. urophylla* × *E. grandis* reached maximum activity at T4, 2.36 times that of T1, indicating that appropriate SNP concentrations help increase APX activity.

2.3 Effects of Treatments on Osmotic Adjustment Substance Contents in Eucalyptus Seedlings

As shown in Figure 4 [Figure 4: see original paper], compared with CK, T1 significantly increased soluble protein content in *E. urophylla* seedlings. With increasing SNP concentration, soluble protein content in leaves of all four species showed a trend of first increasing then decreasing. *E. grandis* reached maximum content at T5, increasing significantly by 8.04% compared with T1 ($P < 0.05$). *E. urophylla* reached maximum content at T5, increasing significantly by 15.37% ($P < 0.05$). *E. tereticornis* reached maximum content at T5, increasing significantly by 10.96% ($P < 0.05$). *E. urophylla* × *E. grandis* reached maximum content at T2, increasing significantly by 5.02%. All four species reached maximum values at either T2 or T5, indicating that appropriate SNP concentrations help increase soluble protein content in Eucalyptus seedlings.

Compared with CK, T1 significantly increased soluble sugar content in *E. urophylla* and *E. tereticornis* but had no significant effect on *E. grandis* and *E. urophylla* × *E. grandis*. With increasing SNP concentration, soluble sugar content in leaves of all four species showed a trend of first increasing then decreasing. *E. grandis* reached maximum content at T3, increasing significantly by 13.51% compared with T1 ($P < 0.05$). *E. urophylla* reached maximum content at T2, increasing significantly by 33.20% ($P < 0.05$). *E. tereticornis* reached maximum content at T3, increasing significantly by 31.11% ($P < 0.05$). *E. urophylla* × *E. grandis* reached maximum content at T4, increasing significantly by 8.21%. All four species reached maximum values at T2, T3, or T4, indicating that appropriate SNP concentrations help increase soluble sugar content in Eucalyptus seedlings.

2.4 Principal Component Analysis of Four Eucalyptus Species

To examine differences in nine physiological indicators among the four Eucalyptus species, we performed principal component analysis to reduce the dimensionality of response variables. As shown in Table 3 , under single aluminum stress, two principal components were retained, with the first and second components contributing 42.90% and 37.11% respectively, for a cumulative contribution of 80.01%. The first principal component was primarily influenced by SOD, MDA, and CAT, while the second was mainly affected by O_2^- production rate, soluble sugar, and soluble protein. Under SNP treatment combined with aluminum stress, the first, second, and third principal components contributed 27.96%, 21.58%, and 15.20% respectively, with a cumulative contribution of 64.74%. The first principal component was primarily influenced by APX, SOD, CAT, and soluble sugar; the second by O_2^- production rate, MDA, and soluble protein; and the third by H_2O_2.

As shown in Figure 5 [Figure 5: see original paper], under single aluminum stress, the four Eucalyptus species were clearly separated (Figure 5A), with *E. urophylla* showing the strongest aluminum resistance, followed by *E. urophylla* × *E. grandis* and *E. grandis*, while *E. tereticornis* showed the weakest resistance. Under aluminum stress with SNP treatment, the points representing the four species were relatively concentrated (Figures 5B, 5C, 5D), indicating that aluminum resistance tended to become consistent across all four species with NO addition.

Discussion

3.1 Effects of NO Addition on Eucalyptus Response to Aluminum Stress

Eucalyptus seedling responses to aluminum stress primarily manifest in cell membrane systems, protective enzyme activities, osmotic adjustment substances, and metabolic activity (Li Tangkan, 2020). This study selected representative indicators from these aspects to explore the protective effects of exogenous SNP on four Eucalyptus species under aluminum stress. Aluminum stress generates large amounts of ROS in plants, causing oxidative stress that damages biological macromolecules such as lipids, proteins, and DNA, leading to cell death and affecting plant physiological status (Guo Peng, 2018). ${O_2}^-$ and H_2O_2 are primary indicators of plant oxidative damage, while MDA is a product of membrane lipid peroxidation. These are important physiological indicators reflecting plasma membrane oxidative stress levels, with their contents positively correlated with membrane oxidative damage (Yamamoto, 2019). When ROS accumulate in large quantities, plants activate antioxidant systems for scavenging to maintain cellular redox homeostasis.

Our results showed that compared with the control group, aluminum stress treatment significantly increased ${O_2}^-$ production rate and MDA accumulation in all four Eucalyptus species. Addition of appropriate SNP concentrations (50–100 $\mathrm{mol \cdot L^{-1}}$ for *E. grandis* and *E. urophylla*, 100–200 $\mathrm{mol \cdot L^{-1}}$ for *E. tereticornis*, and 50–200 $\mathrm{mol \cdot L^{-1}}$ for *E. urophylla* × *E. grandis*) effectively increased SOD, POD, CAT, and APX activities and soluble protein and soluble sugar contents while reducing ${O_2}^-$ production rate and MDA content, thereby decreasing plasma membrane peroxidation damage. This indicates that appropriate SNP concentrations under aluminum stress can enhance antioxidant enzyme activity and strengthen ROS scavenging capacity to improve plant aluminum tolerance, consistent with findings from Hou Wenjuan et al. (2019). However, application of 800 $\mathrm{mol \cdot L^{-1}}$ SNP increased ${O_2}^-$ production rate and soluble protein content while decreasing soluble sugar content and antioxidant enzyme activities such as SOD in all four species. This may occur because, besides acting as a signaling molecule in plant physiological processes, NO is also a reactive nitrogen species. High SNP concentrations cause excessive accumulation of reactive nitrogen in plants, leading to nitrosative stress. The dual stress of aluminum toxicity and nitrosative stress severely damages the antioxidant system and inhibits antioxidant enzyme activities. Thus, NO as an important gaseous signaling molecule has dual effects: appropriate exogenous NO application alleviates physiological damage in Eucalyptus seedlings under aluminum stress, while high concentrations produce inhibitory effects. These results align with studies on watermelon (Xiao Jiaxiong et al., 2021), *E. urophylla* × *E. grandis* DH3229 (Hou Wenjuan et al., 2019), and *Castanopsis hystrix* (Li Lin et al., 2020). To verify the extent of nitrosative stress effects, we will measure and analyze specific nitrosative stress indicators (glutathione reductase (GSNOR) activity, peroxynitrite anion ($ONOO^-$), and NO distribution in plant tissues) in subsequent experiments to further validate our experimental conclusions.

3.2 Key Indicators for Characterizing Aluminum Tolerance in Eucalyptus

Under single aluminum stress, PC1 primarily represented antioxidant capacity and membrane lipid peroxidation degree, while PC2 represented osmotic adjustment substance content. Under aluminum stress with NO addition, PC1 primarily represented antioxidant capacity, PC2 represented membrane lipid peroxidation degree, and PC3 represented ROS accumulation degree. In summary, SOD, MDA, CAT, O_2^-, soluble protein, and soluble sugar can serve as key indicators for evaluating aluminum tolerance in Eucalyptus. When Eucalyptus experiences aluminum stress, large amounts of ROS (including O_2^- and H_2O_2) accumulate in vivo. Plants primarily cope by increasing antioxidant enzyme activities to scavenge ROS and by biosynthesizing soluble compounds (including soluble sugars and soluble proteins) to regulate cellular osmotic conditions and maintain membrane integrity and function (Benzarti et al., 2014).

3.3 Interspecific Differences in Aluminum Tolerance and Beneficial Effects of NO on Aluminum Stress

Under single aluminum stress, *E. urophylla* showed the strongest aluminum resistance, followed by *E. urophylla* × *E. grandis* and *E. grandis*, while *E. tereticornis* showed the weakest resistance. This is primarily because *E. urophylla* exhibited greater increases in APX activity under aluminum stress, enhancing antioxidant capacity, and showed larger increases in osmotic adjustment substances, providing better membrane protection and lower membrane lipid peroxidation. Although *E. tereticornis* also had relatively high antioxidant enzyme activities and osmotic adjustment capacity, its MDA content was also high, suggesting that *E. tereticornis* responds sensitively to aluminum stress with active resistance responses but is limited by inherent deficiencies, resulting in higher membrane lipid peroxidation and weaker resistance. This is generally consistent with our previous research results (Liang et al., 2022).

Interspecific differences in aluminum tolerance among Eucalyptus species may be related to their adaptive evolutionary history. *E. urophylla* is native to volcanic-derived soils in eastern Indonesian islands (7°30 to 10°S) (Sein & Mitlöhner, 2011), where soils are rich in bauxite, iron ore, and aluminum/iron-humus complexes (Ugolini & Dahlgren, 2002; Yatno & Zauyah, 2008). Long-term selection in aluminum-rich environments has led to strong aluminum resistance in *E. urophylla* (Steane et al., 2011). *E. grandis* is native to eastern Australia (17 to 32°S) (Burgess, 1988). *E. tereticornis* has the widest natural distribution, ranging from Papua New Guinea to southern Australia (6–38°S) (González et al., 2021). Large latitudinal differences cause direct or indirect anthropogenic and environmental effects on soil conditions (such as pH), potentially resulting in strong stress plasticity in *E. tereticornis.* In this experiment, we found *E. tereticornis* had the weakest aluminum resistance, but previous experiments showed its aluminum tolerance was intermediate between *E. urophylla* and *E. grandis* (Liang et al., 2022), possibly due to individual differences among different seedling batches. Overall, differences in aluminum tolerance among *E. grandis*, *E. urophylla*, and *E. tereticornis* may be attributed to their different habitats. As a hybrid of *E. urophylla* and *E. grandis*, *E. urophylla* × *E. grandis* shows aluminum resistance intermediate between its parents. The hybrid exhibits characteristics similar to its maternal parent (*E. urophylla*), suggesting that aluminum stress resistance is heritable, controlled by few genes, and largely inherited in hybrid species. Genetic control of aluminum tolerance has been identified in many plants, and aluminum-tolerant crop varieties have been bred (Coelho et al., 2019; Miftahudin et al., 2021; Sara et al., 2020; Zhao et al., 2018). Many countries have conducted research on Eucalyptus hybrid improvement, breeding, and planting, developing improved varieties with high yield, high resistance, and high adaptability (Zhu et al., 2018). This indicates that breeding aluminum-tolerant Eucalyptus genotypes is feasible and promising. Notably, although this study found that the hybrid variety has strong aluminum tolerance similar to its maternal parent, the paternal parent has lower aluminum tolerance. Therefore, reciprocal crossing experiments are needed to further verify the genetic characteristics of aluminum tolerance in Eucalyptus.

Our results demonstrate that exogenous NO can reduce aluminum stress damage and improve aluminum tolerance by activating antioxidant enzyme activities and increasing osmotic adjustment substances. Previous studies found that in ryegrass (Wu Ya, 2019) and tobacco (Liu Qiang et al., 2016), exogenous NO had more pronounced alleviating effects on aluminum-sensitive genotypes (ryegrass Nagahahikari and tobacco Yunyun 105) than on tolerant genotypes under aluminum stress, showing stronger aluminum tolerance. Our experiments revealed similar results: under exogenous NO treatment, aluminum tolerance among the four Eucalyptus species became relatively concentrated. This may be because NO acts like a buffer, improving the aluminum resistance of originally weaker species (*E. grandis* and *E. tereticornis*, the sensitive types) while having little effect on the originally stronger *E. urophylla* (the tolerant type), ultimately resulting in similar aluminum resistance across all four species under NO treatment.

Appropriate NO concentrations (50–200 $\text{mol} \cdot \text{L}^{-1}$) can improve Eucalyptus aluminum resistance by enhancing antioxidant enzyme activities and osmotic adjustment substance contents and reducing MDA content under aluminum stress. However, excessive NO concentrations (≥ 800 $\text{mol} \cdot \text{L}^{-1}$) cause stress responses. Nitric oxide significantly improves aluminum tolerance in sensitive Eucalyptus species but has less effect on tolerant species, ultimately resulting in similar aluminum resistance across all four species under NO treatment. SOD, MDA, CAT, O_2^-, soluble protein, and soluble sugar can serve as key indicators for evaluating aluminum tolerance in Eucalyptus.

References

BENZARTI M, REJEB KB, MESSEDI D, et al., 2014. Effect of high salinity on *Atriplex portulacoides*: Growth, leaf water relations and solute accumulation in relation with osmotic adjustment[J]. S Afr J Bot, 95: 70-77.

BURGESS IP. 1988. Provenance trials of *Eucalyptus grandis* and *E. saligna* in Australia[J]. Silvae Genet, 37(5-6): 221-227.

CHEN JX, WANG XF, 2006. Experimental guidance in plant physiology[M].

Guangzhou: South China University of Technology Press: 54-127.

COELHO CD, BOMBARDELLI RGH, SCHULZE GS, et al., 2019. Genetic control of aluminum tolerance in tropical maize germplasm[J]. Bragantia, 78(1): 71-81.

GUO P, 2018. Molecular and physiological mechanism of aluminum-tolerance and sulfur-mediated-alleviation of aluminum-toxicity in Citrus[D]. Fuzhou: Fujian Agriculture and Forestry University.

GONZÁLEZ A, CABRERA MLÁ, HENRIQUEZ MJ, et al., 2012. Cross talk among calcium, hydrogen peroxide, and nitric oxide and activation of gene expression involving calmodulins and calcium-dependent protein kinases in *Ulva compressa* exposed to copper excess[J]. Plant Physiol, 158(3): 1451-1462.

GONZÁLEZ M, BALMELLI G, RESQUIN F. 2021. Performance of *Eucalyptus tereticornis* provenances in subtropical climate[J]. Agrociencia Uruguay, 25(1): 322-334.

HOU WJ, LUO WJ, WEI J, et al., 2019. Response of *Eucalyptus* DH3229 seedlings to SNP-acid aluminum nitrate interactions[J]. Guihaia, 39(4): 437-445.

HUANG LP, ZHOU ZL, WU YY, et al., 2022. Research progress of non-volatile chemical components from *Eucalyptus* genus plants and their pharmacological activities[J]. Guihaia, 42(4): 531-542.

HUANG QQ, 2021. Study on the change of soil phosphorus form and its bioavailability in orchard grass[D]. Yangling Demonstration Zone: Northwest A & F University.

KONG XS, YI XF, 2008. Experimental techniques in plant physiology[M]. Beijing: China Agriculture Press: 124-126.

LI HS, 2000. Principles and techniques of plant physiological and biochemical experiments[M]. Beijing: Higher Education Press: 164-261.

LI L, 2020. Effects of SNP-Al interaction on the growth and physiology of *Phoebe bournei* seedlings[D]. Nanning: Guangxi University.

LI L, FANG AH, KANG YC, et al., 2020. Effects of SNP-$AlCl_3$ interaction on growths of *Castanopsis hystrix* seedlings and comprehensive evaluation[J]. Guangxi Forestry Science, 49(02): 186-191.

LI TK, 2020. Operation mode and evaluation of antioxidant system in root tip of four *Eucalyptus* seedlings under aluminum stress[D]. Nanning: Guangxi University.

LI Y, 2017. Regulation of NO and glucose-6-phosphate dehydrogenase in response to aluminum stress in soybean[D]. Xinxiang: Henan Normal University.

LIANG JX, 2020. Al stress on root tip antioxidant system of two *Eucalyptus* seedlings under H_2O_2 pretreatment[D]. Nanning: Guangxi University.

LIANG YH, 2022. Physiological response characteristics of four species of *Eucalyptus* seedlings to aluminum stress and the mechanism of alleviating effect under hydrogen peroxide pretreatment[D]. Nanning: Guangxi University.

LIANG YH, BAI TD, LIU B, et al., 2022. Different antioxidant regulation mechanisms in response to aluminum-induced oxidative stress in *Eucalyptus* species[J]. Ecotox Environ Safe, 174(2): 1137-1148.

LIU Q, HE ZG, LIU ZHW, et al., 2016. Effects of exogenous nitric oxide on light energy utilization, photoprotection system and active oxygen metabolism of tobacco leaves under aluminum stress[J]. Journal of Hunan Agricultural University, 42(6): 615-621.

LIU Q, LIU ZHW, HU C, et al., 2017. Effects of exogenous nitric oxide on respiration and active oxygen metabolism of tobacco leaves under aluminum stress[J]. Henan Agricultural Sciences, 46(1): 42-47.

LU MY, 2014. Cell biological mechanisms of aluminum induced on root apex of different aluminum-tolerant fast-growing *Eucalyptus* clones[D]. Nanning: Guangxi University.

MIFTAHUDIN M, INDRIYANI RD, HUDA FM, et al., 2021. OsGERLP: A novel aluminum tolerance rice gene isolated from a local cultivar in Indonesia[J]. Plant Physiol Bioch, 162(2): 86-99.

PEREIRA LB, MAZZANTI C, CARGNELUTTI D, et al., 2011. Differential responses of oat genotypes: oxidative stress provoked by aluminum[J]. Biometals, 24(1): 73-83.

R Core T, 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing.

SARA F, ALI FB, MAHDI NN, et al., 2020. Genetic control of some plant growth characteristics of bread wheat (*Triticum aestivum* L.) under aluminum stress[J]. Genes Genom, 42(3): 245-261.

SEIN CC, MITLÖHNER R. 2011. *Eucalyptus urophylla* S.T. Blake: ecology and silviculture in Vietnam[M]. Jakarta: Center for International Forestry Research (CIFOR), 16.

STEANE D, NICOLLE D, SANSALONI C, et al., 2011. Population genetic analysis and phylogeny reconstruction in *Eucalyptus* (Myrtaceae) using high-throughput, genome-wide genotyping[J]. Mol Phylogenet Evol, 59(1): 206-224.

UGOLINI FC, DAHLGREN RA. 2002. Soil development in volcanic ash[J]. Glob Environ Res, 6(2): 69-86.

WANG HH, ZHANG YY, LIU WW, 2019. Mitigating effect of nitric oxide on root growth inhibition of soybean under aluminum stress[J]. Jiangsu Agricultural Sciences, 47(18): 122-125.

WANG XK, 2000. Principle and technology of plant physiology and biochemistry[M]. Beijing: Higher Education Press: 118-279.

WEI YH, NONG CL, ZHOU MS, et al., 2021. Application of organic waste compost in *Eucalyptus* container seedlings[J]. Journal of Central South University of Forestry and Technology, 41(10): 83-89.

WU Y, 2019. Study on relieving effects of exogenous salicylic acid and NO on ryegrass under aluminum stress[D]. Yangzhou: Yangzhou University.

XIAO JX, ZHENG KM, MA JY, et al., 2021. Effects of exogenous NO on growth and physiological characteristics of watermelon seedlings under aluminum stress[J]. Journal of Agro-Environment Science, 40(8): 1650-1658.

XIE YJ, 2015. Real eucalyptus[M]. Beijing: China Forestry Publishing House: 2-10.

XIONG J, AN L, LU H, et al. 2009. Exogenous nitric oxide enhances cadmium tolerance of rice by increasing pectin and hemicellulose contents in root cell wall[J]. Planta, 230(4): 755-765.

XIONG J, FU G, TAO L, et al. 2010. Roles of nitric oxide in alleviating heavy metal toxicity in plants[J]. Arch Biochem Biophys, 497(1): 13-20.

YAMAMOTO Y. 2019. Aluminum toxicity in plant cells: Mechanisms of cell death and inhibition of cell elongation[J]. Soil Sci Plant Nutr, 65(1): 41-55.

YATNO E, ZAUYAH S. 2008. Properties and management implications of soils formed from volcanic materials in Lembang area, West Java[J]. Indones J Agric Sci, 9(2): 44-54.

YI S, ZHIHUI Z, KAIJUN X. 2015. Evaluation of cobalt oxide, copper oxide and their solid solutions as heterogeneous catalysts for Fenton-degradation of dye pollutants[J]. RSC Adv, 5(111): 91846-91854.

YIN L, WANG S, ELTAVEB AE, et al. 2010. Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco[J]. Planta, 231(3): 609-621.

YU M, LAMATTINA L, SPOEL SH, et al., 2014. Nitric oxide function in plant biology: a redox cue in deconvolution[J]. New Phytol, 202(4): 1142-1156.

ZHAO H, HUANG W, ZHANG Y, et al. 2018. Natural variation of *CsSTOP1* in tea plant (*Camellia sinensis*) related to aluminum tolerance[J]. Plant Soil, 431(1-2): 71-87.

ZHU YA, XU JM, LI GY, et al. 2018. Genetic parameters for wood properties in a reciprocal factorial mating design between *Eucalyptus urophylla* and *E. camaldulensis*[J]. Forest Res, 31(6): 121-129.

Note: Figure translations are in progress. See original paper for figures.

Source: ChinaXiv — Machine translation. Verify with original.